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ARYL HYDROCARBON HYDROXYLASE INDUCTION IN MAMMALIAN LIVER-DERIVED CELL CULTURES

EFFECTS OF VARIOUS METABOLIC INHIBITORS ON THE ENZYME ACTIVITY IN HEPATOMA CELLS*

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Abstract— Aryl hydrocarbon (benzo[a]pyrene) hydroxylase induction in one mouse hepatoma and two rat hepatoma cell lines was characterized with respect to optimal growth requirements and optimal inducing concentrations of polycyclic hydrocarbons, phenobarbital, biogenic amines, and numerous other hydrophobic compounds. Cordycepin, ethidium bromide and puromycin aminonucleoside treatment of the mouse tumor line Hepa-1 produce strikingly different effects on the kinetics of hydroxylase induction, when the inducers benz[a]anthracene, phenobarbital and iso-proterenol are compared. Without any of these 'usual inducers' present in the mouse hepatoma cell line, 0·50 to 5·0 mM puromycin aminonucleoside or 40 nM actinomycin-D causes large increases in the hydroxylase activity (at a time when gross RNA synthesis is virtually 100 per cent inhibited and gross protein synthesis is more than 50 per cent inhibited). This aminonucleoside- or actinomycin-D-mediated induction process induction, when the inducers benz[a]anthracene, phenobarbital and isoproterenol are compared. With-without prior treatment with the usual inducers and hence presumably without prior accumulation of putative induction-specific RNA: moreover, this induction process apparently requires little, or no, simultaneous RNA synthesis during exposure to the aminonucleoside or actinomycin-D. In contrast, when primary cultures derived from normal rat liver are treated with similar high concentrations of the aminonucleoside, no induction of the hydroxylase activity occurs. This result suggests that the effect of puromycin aminonucleoside on hydroxylase-specific mRNA synthesis, processing or stabilization is very different between the hepatoma cell line and normal fetal rat primary hepatocytes in culture.

Recent reports from this laboratory [1–4] show that aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity†‡ accumulates (or is 'induced') in cultured fetal rat liver cells treated with polycyclic hydrocarbons such as 3-methylcholanthrene and benz[a]anthracene, the insecticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (*p,p'*-DDT), phenobarbital or biogenic amines such as tryptamine, isoproterenol, norepinephrine and histamine. 3-Methylcholanthrene, phenobarbital and biogenic amines all cause increases in hydroxylase activity that are additive or synergistic when two or three of these types of inducers are combined in the culture medium [1, 2, 4], indicating different modes of action for these different classes of inducers of mono-oxygenase activities [5, 6]. The action of polycyclic hydrocarbons, phenobarbital and biogenic amines on hydroxylase induction appears to involve transcription [3], since each of the induction processes is inhibited when actinomycin-D is added simultaneously with the inducer initially [3, 4]. There also appears to be a post-translational effect, because each of these three types of inducers can slow the regular rate of decay of induced hydroxylase activity [3, 4]. Further, if the enzyme activity is first maximally induced by any one of these three types of inducers and then RNA synthesis is blocked by actinomycin-D, a different inducer subsequently added can direct, presumably at the post-transcriptional level, a further rise in the hydroxylase activity [4]. The advantages of established cell lines (from rat or mouse hepatomas or from adult rat liver) in studying hydroxylase induction [7] and cytochrome P_1-450 formation [8] have been recently demonstrated; furthermore, the extremely low basal hydroxylase activity of the H-4-II-E cell line and the sensitive rise in inducible enzyme activity in response to inducers suggest the potential usefulness of this tumor cell line in assaying minute amounts of various foreign compounds [9] which are inducers.

The compounds cordycepin [10], ethidium bromide [11], puromycin aminonucleoside [12] and actinomycin-D [13] are known to inhibit RNA synthesis by various rather specific mechanisms, as demonstrated in the references cited. In this report,

* Previous papers in this series have appeared [1–4, 7–9].

† With benzo[a]pyrene as the substrate *in vitro*, 'aryl hydrocarbon hydroxylase activity' is equated with the rate of formation of 3-hydroxybenzo[a]pyrene and probably other phenols having similar wavelengths of fluorescent activation and emission. These phenols may be formed either by a direct hydroxylation or in a two-step process via an arene oxide.

‡ The abbreviations used are: the hydroxylase, aryl hydrocarbon (benzo[a]pyrene) hydroxylase; metyrapone, 2-methyl-1,2,3,3-pyridyl-1-propanone; and cordycepin, 3'-deoxyadenosine.

we first describe the optimal conditions for studying the hydroxylase induction in three hepatoma-derived cell lines. Second, with the use of these various inhibitors commonly used in tissue culture studies, we show differences between these 'classes' of mono-oxygenase inducers (i.e. polycyclic hydrocarbons, phenobarbital and biogenic amines) in an attempt to elucidate further the subcellular mechanisms involved in the enzyme induction process.

EXPERIMENTAL PROCEDURE

Materials

The tissue culture materials [2–4, 7] and all of the chemicals [4, 14] used in this study were obtained from the sources cited. The compounds cordycepin, cycloheximide and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, Mo.); puromycin aminonucleoside from Nutritional Biochemical Corp. (Cleveland, Ohio); and actinomycin-D from CalBiochem Co. (Los Angeles, Calif.). Nominally labeled L-[3-^{3}H]phenylalanine (12·69 Ci m-mole) and uniformly labeled [^{14}C]uridine (400 mCi m-mole) were purchased from New England Nuclear (Boston, Mass.). Hepa-1, a mouse cell line derived from the transplantable BW 7756 hepatoma originally produced in the C57L/J mouse [15], was kindly provided in 1971 by Dr. Gretchen Darlington, Department of Biology, Yale University (New Haven, Conn.). H-4-II-E, a rat cell line derived [16] from Reuber hepatoma H-35, was generously given to us by Dr. E. Brad Thompson, National Cancer Institute (Bethesda, Md.). MH_1C_1, a clone of epithelial cells from the transplantable Morris hepatoma 7795 originally produced in the Buffalo strain of rat [17], was purchased from the American Type Culture Collection Cell Repository (Rockville, Md.). Taconic Farms, Inc. (Germantown, N. Y.) provided us with pregnant Sprague–Dawley rats.

Methods

Preparation of compounds in growth medium and handling of the cell lines. For those chemicals that did not dissolve readily in the culture medium, the compounds were initially dissolved in a minimal amount of acetone, ethanol or dimethylsulfoxide and then added to the growth medium. These organic solvents at concentrations of 0·5% or less in the medium were shown not to have any effect on the parameters under investigation. Because of the propensity for 3-methylcholanthrene and benz[a]-anthracene to bind to glass or plastic, the actual concentration of these polycyclic hydrocarbons dissolved in the medium was always determined by spectrophotofluorometry. The possible toxic effects of the various compounds at every concentration tested were evaluated daily with the use of light microscopy, protein determinations when the cultures were harvested, and frequent 30-min checks on gross RNA and protein synthesis. Addition of the inducing compounds was always carried out about 48 hr after the plating of the hepatoma cell lines or the primary rat liver cultures [2–4, 7–9]. For any exposure lasting more than 24 hr, the medium was replaced with fresh medium containing the compound(s) every 24 hr. In the table and in all the figures a value for specific hydroxylase activity represents the average of duplicate determinations of enzyme activity and protein concentration on each of two 60-mm tissue culture dishes. Each experiment was performed two to five times, in order to ensure reproducibility.

Enzyme assay. Both the hydroxylase activity and protein concentration were determined in duplicate for the homogenate from cells scraped from one cell culture dish 60 mm in diameter [2, 4]. The 1·00-ml reaction mixture includes 50 μmoles potassium phosphate buffer, pH 7·5, 0·36 μmole NADH, 600 μg albumin, 3 μmoles $MgCl_2$, 200–600 μg protein of cellular homogenate to be assayed, and 80 nmoles benzo[a]-pyrene and is incubated for 30 min [18]. One unit of aryl hydrocarbon hydroxylase activity is defined [1] as that amount of enzyme catalyzing min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole 3-hydroxybenzo-[a]pyrene recrystallized standard. The specific activity is always expressed in units mg of protein of the total cellular homogenate.

Gross RNA and protein synthesis. The incorporation of [^{14}C]uridine and [^{3}H]phenylalanine into perchloric acid-precipitable material was used as an estimation of total cellular RNA and protein syntheses respectively. To the cells of one 60-mm tissue culture dish was added 0·5 μCi of each labeled compound for a 30-min pulse, as described previously in detail [4]. After a 30-min exposure, the cellular macromolecules were precipitated with 2 ml of cold 0·5 M perchloric acid. The precipitate was centrifuged at 2000 g for 5 min and washed twice with 3 ml of cold 0·025 M perchloric acid. The precipitate was then dissolved in 1 ml Nuclear Chicago Solubilizer. Ten ml Liquifluor (Amersham/Searle) was added, and the radioactivity of both ^{14}C and ^{3}H in cultures exposed to the radioisotopes for zero time was subtracted from that of each experimental sample. The specific radioactivity for three identical tissue culture dishes harvested at the same time point varied less than 15 per cent. The gross RNA and protein synthesis of any experimental cultures were compared with those in control cultures receiving no metabolic inhibitors and, therefore, are expressed throughout this report as 'per cent of the control.'

RESULTS

Optimal conditions for hydroxylase induction. Table 1 summarizes the basal and inducible hydroxylase activities in the three established cell lines, when exposed to the inducers 3-methylcholanthrene, phenobarbital or tryptamine under various culture conditions. We include these data in this report principally as information to others who might wish to study various aspects of drug metabolism in hepatoma cell cultures. A considerable amount of induction in the absence of any serum was found; similar results had been reported [2] in fetal rat primary liver cultures. From this preliminary survey, the following types of medium and the percents and types of serum were chosen for the remainder of the studies in this report: MAB medium [7] with 10% fetal calf serum for Hepa-1 and Eagle's medium with 10% each of calf and fetal calf sera for H-4-II-E. Because the MH_1C_1

Table 1. Basal and 'inducible' hydroxylase activity in Hepa-1, H-4-II-E, and MH_1 C_1 cultures studied under various growth conditions*

			Aryl hydrocarbon hydroxylase specific activity†											
			Hepa-1				H-4-II-E				MH_1C_1			
Medium	Serum	Serum (%)	Control medium	3-Methylcholanthrene	Phenobarbital	Tryptamine	Control medium	3-Methylcholanthrene	Phenobarbital	Tryptamine	Control medium	3-Methylcholanthrene	Phenobarbital	Tryptamine
MAB	None		23	110	91	100	2	15	5	31	11	81	18	41
	Fetal calf	10	34	160	150	160	3	66	8	26	11	110	24	66
	Fetal calf	20	39	210	190	140	4	59	12	46	31	99	37	86
	Fetal calf plus calf	10 10	32	220	220	140	3	38	8	36	15	97	28	120
	Calf	10	21	230	180	170	2	35	9	34	12	130	26	61
	Calf	20	24	300	170	120	3	45	8	36	16	110	28	81
Eagle's	None		5	35	32	29	2	12	3	26	5	74	6	54
	Fetal calf	10	15	46	52	54	2	50	17	37	16	110	26	120
	Fetal calf	20	21	66	69	59	3	55	18	41	16	93	35	96
	Fetal calf plus calf	10 10	11	77	77	64	4	98	30	57	10	56	25	49
	Calf	10	7	51	43	57	4	84	29	72	9	82	21	63
	Calf	20	6	90	73	54	5	86	23	50	14	67	19	60

* Cultures were grown, until the beginning of logarithmic growth, in medium and calf serum known to be favorable for cell division: MAB medium plus 10% fetal calf serum for Hepa-1, Eagle's medium with 10% fetal calf serum and 10% calf serum for H-4-II-E, and Eagle's medium plus 10% calf serum for MH_1 C_1. The surface of the cells was washed several times with the 'test medium,' and the cultures were then grown for 24 hr in the 'test medium,' which was MAB or Eagle's medium to which different concentrations of fetal calf or calf serum had been added. The inducer 2·0 μM 3-methylcholanthrene, 2·0 mM phenobarbital, 0·50 mM tryptamine or control medium alone in the 'test medium' was added for a second 24 hr period, after which the enzyme activity was determined.

† Expressed in units/mg of cellular protein.

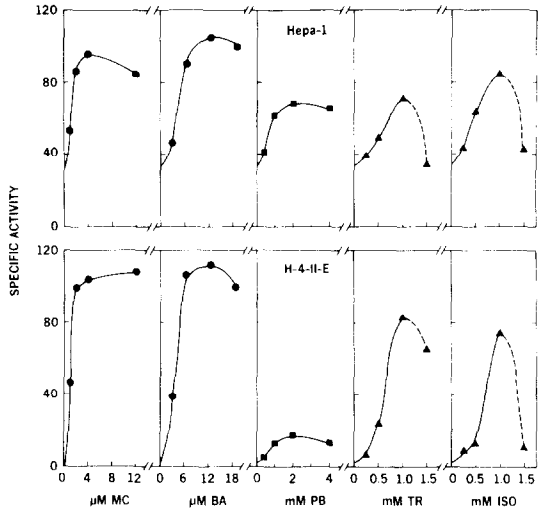

Fig. 1. Inducible hydroxylase activity as a function of inducer concentration in the growth medium. Hepa-1 or H-4-II-E cultures were treated with the inducing compounds 3-methylcholanthrene (MC), benz[a]anthracene (BA), phenobarbital (PB), tryptamine (TR) or isoproterenol (ISO) for 48 hr. Cytotoxicity was evident at 1·5 mM tryptamine and 1·5 mM isoproterenol.

cultures grew most slowly (generation time of 35–45 hr) of the three lines investigated, we chose not to use MH_1C_1 further in this study. H-4-II-E cultures have a doubling time between 18 and 24 hr; the Hepa-1 line has a generation time between 24 and 32 hr.

Optimal inducer concentration. Figure 1 shows the optimal inducing concentrations of the compounds most commonly used in this study. The results for MH_1C_1 cultures (data not shown) were quite similar to those for Hepa-1 and H-4-II-E cell lines.

Figure 2 shows that the combination of phenobarbital plus 3-methylcholanthrene in the medium induces the hydroxylase activity to levels which are approximately the sum of those induced by either phenobarbital or 3-methylcholanthrene alone. The kinetics of hydroxylase induction have been illustrated previously [7]: the maximal level of induced enzyme activity is usually attained in 3 days for Hepa-1 cultures and in 2 days for H-4-II-E cells. This fact is the reason for choosing (see Fig. 2) the time points 48 and 72 hr for Hepa-1 and 24 and 48 hr for H-4-II-E cultures. This 'additive' effect for phenobarbital plus 3-methylcholanthrene had been found before in the intact animal [5] and in fetal rat primary hepatocyte cultures [1, 2] and indicates some fundamental difference in the mechanism of action by which these two 'classes' of mono-oxygenase inducers [5, 6] exert their effect on the induction process.

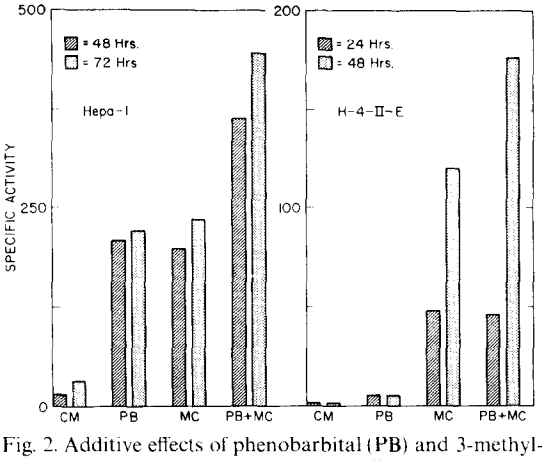

Fig. 2. Additive effects of phenobarbital (PB) and 3-methylcholanthrene (MC) in the culture medium on inducible hydroxylase activity. The Hepa-1 cells were assayed for enzyme activity after 48 and 72 hr of treatment with control medium (CM), 2·0 mM phenobarbital, 2·0 μm 3-methylcholanthrene or 2·0 mM phenobarbital plus 2·0 μM 3-methylcholanthrene. The H-4-II-E cultures were assayed for enzyme activity after 24 and 48 hr of treatment with control medium, 2·0 mM phenobarbital, 4·0 μM 3-methylcholanthrene or 2·0 mM phenobarbital plus 4·0 μM 3-methylcholanthrene.

Induction of hydroxylase activity by numerous hydrophobic compounds. Figure 3 shows five other 'inducers' of the hydroxylase activity in Hepa-1 and H-4-II-E cell lines. Subtle differences between the two cell lines in fold induction and in the maximal enzyme activity reached are illustrated. The two most significant differences shown are: (1) a much greater response of the induction process to metyrapone in H-4-II-E cells than in Hepa-1 cells, and (2) a basal hydroxylase activity 20–30 times higher in Hepa-1 cultures than in H-4-II-E (also seen in Table 1 and Figs. 1 and 2). Because we wished to study changes in the basal, as well as the induced, hydroxylase activity and because phenobarbital-induced hydroxylase activity was considerably higher in Hepa-1, the Hepa-1 rather

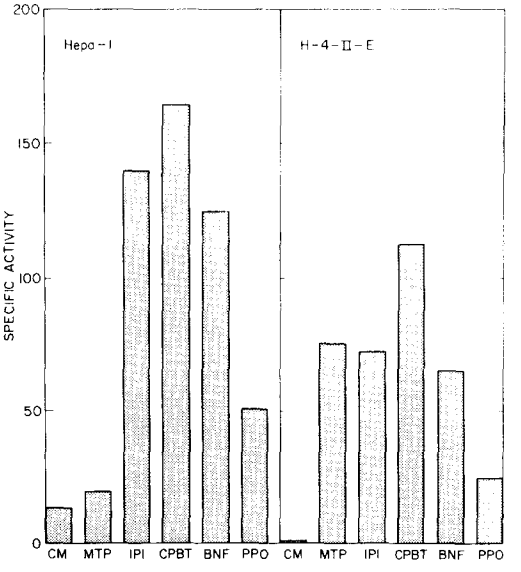

Fig. 3. Induction of hydroxylase activity in Hepa-1 and H-4-II-E cultures by 500 μM metyrapone (MTP), 100 μM 1-(2-isopropylphenyl)imidazole (IPI), 1·0 mM 2-(4′-chlorophenyl)benzothiazole (CPBT), 50 μM β-naphthoflavone (BNF) and 100 μM 2,5-diphenyloxazole (PPO). Control cells remained in control medium (CM) only. The values shown are taken from cells exposed to optimal inducing concentrations for each compound for 48 hr, at which time the enzyme activity was maximal or nearly maximal. Cytotoxicity, as determined by the parameters set forth in 'Experimental Procedure,' was not observed at these optimal inducing concentrations for any of these compounds.

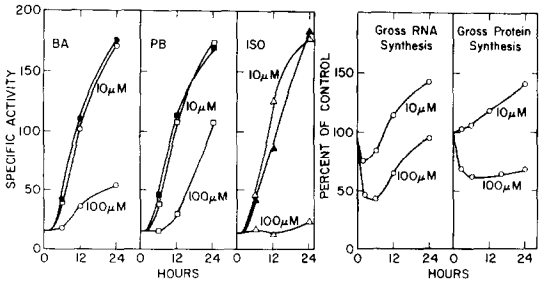

Fig. 4. Effect of cordycepin on hydroxylase induction in Hepa-1 cultures by 1·3 μM benz[a]anthracene (BA, ●), 2·0 mM phenobarbital (PB, ■) or 1·0 mM isoproterenol (ISO, ▲) and on gross RNA and protein synthesis. Concentrations of 10 and 100 μM cordycepin in the presence of each inducer are shown with open symbols. Cordycepin alone had no effect on the control enzyme activity. In this and in subsequent figures, none of the inducers, benz[a]anthracene, phenobarbital or isoproterenol, affected the changes in gross RNA synthesis (i.e. [^{14}C]uridine incorporation into trichloroacetic acid-precipitable material) and gross protein synthesis (i.e. [^{3}H]phenylalanine incorporation into trichloroacetic acid-precipitable material) produced by the metabolic inhibitor under study.

than the H-4-II-E cell line was used for the remainder of the studies in this report.

Cordycepin effects. Cordycepin, an analog of adenosine, suppresses the labeling of cytoplasmic mRNA and nuclear HnRNA by blocking the synthesis of the poly(A) segment of mRNA [19–22]. Cordycepin also [17, 18] may function in part by inhibiting mRNA transport from the nucleus [23, 24]. Such short-term studies (e.g. 6 hr) exclude the possibility that cordycepin affects the rate of mRNA translation and ascribe the inhibition of protein synthesis to inhibition of mRNA-poly(A) synthesis and, hence, availability of mRNA to the polysomes. Prolonged cordycepin treatment (e.g. greater than 12 hr) may affect rRNA synthesis and degradation [25]. However, it has been previously shown [3, 26] that rRNA synthesis is probably not critical to aryl hydrocarbon hydroxylase induction over a 24-hr period.

Figure 4 shows that the rise in hydroxylase activity in phenobarbital-treated cultures is relatively resistant to 100 μM cordycepin, compared with benz[a]anthracene- and isoproterenol-treated cultures. Hence, after a 24-hr treatment of the cells with phenobarbital plus 100 μM cordycepin, aryl hydrocarbon hydroxylase induction was about 60 per cent of that by phenobarbital alone. Hydroxylase induction by benz[a]anthracene combined with 100 μM cordycepin was about 20 per cent of that by benz[a]anthracene alone, and induction by isoproterenol was totally blocked by this concentration of cordycepin. During this time gross RNA synthesis was 50 per cent inhibited for 6 hr and fully recovered by 24 hr, and gross protein synthesis was 50 per cent inhibited for the 24-hr period. One interpretation of these data is that the induction process by benz[a]anthracene or isoproterenol in Hepa-1 cells is more sensitive to inhibition of poly(A) synthesis than is induction by phenobarbital.

It is of interest that 10 μM cordycepin caused gross RNA synthesis to be impaired for the first 6–12 hr, after which a significant stimulation occurred, and that 10 μM cordycepin caused gross protein synthesis to be increased significantly above normal. This stimulatory effect was not found with 100 μM cordycepin. This phenomenon might be explained on the basis of labile inhibitors of transcription and translation. At low concentrations of cordycepin, the synthesis of a labile mRNA responsible for such labile protein inhibitors might be preferentially blocked, compared with the relative degree of inhibition of other cellular mRNA's; at high concentrations of cordycepin, this preferential inhibition is not seen because synthesis of the majority of cellular mRNA's is now impaired.

It is of further interest in Fig. 4 that cordycepin, although known to affect mRNA synthesis quite specifically, causes significant changes in gross protein synthesis as well. These important alterations in both gross RNA and protein synthesis were illustrated previously in actinomycin-D- and cycloheximide-treated fetal rat liver cultures [3]. We, therefore, emphasize the necessity to monitor frequently gross RNA and protein synthesis any time a test compound is added to cells in culture. We realize that the monitoring of gross RNA and protein syntheses may not be accurate, if large changes occur in the respective precursor pools. Further studies with this consideration in mind are planned for actinomycin-D, cordycepin, ethidium bromide and puromycin aminonucleoside in Hepa-1 cultures.

Ethidium bromide effects. Ethidium bromide is an effective inhibitor of total nucleic acid synthesis in a variety of organisms [11, 27, 28]. At concentrations as low as 0·6 μM, however, ethidium bromide very selectively inhibits synthesis of the 12S and 21S RNA species in mitochondria of cultured HeLa cells, whereas no inhibition of the incorporation of labeled precursors into nuclear RNA is detectable [29]. Ethidium bromide also inhibits poly(A) synthesis [30]. Large and distinctive differences in the kinetics of hydroxylase induction occurred in ethidium bromide-treated cells (Fig. 5), when the inducers benz[a]anthracene, phenobarbital and isoproterenol were compared. Hydroxylase induction by benz[a]anthracene was inhibited by 1, 2 and 6 μM ethidium bromide only after 6 or 12 hr; the induction process

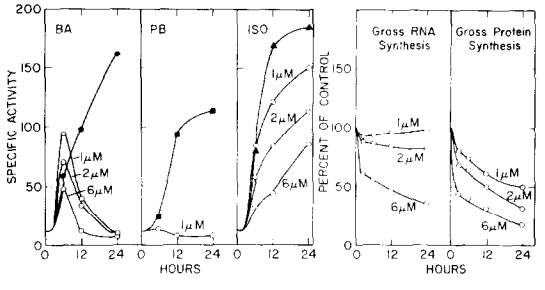

Fig. 5. Effect of ethidium bromide on hydroxylase induction and on gross RNA and protein synthesis in Hepa-1 cultures. Inducing concentrations and the abbreviations for benz[a]anthracene (●), phenobarbital (■) and isoproterenol (▲) are the same as those in Fig. 4. Concentrations of 1·0, 2·0 and 6·0 μM ethidium bromide in the presence of each inducer are shown with open symbols. Ethidium bromide alone had no effect on the control enzyme activity. Gross RNA synthesis (i.e. [^{14}C]uridine incorporation) and gross protein synthesis (i.e. [^{3}H]phenylalamine incorporation) are shown at right.

by phenobarbital was totally blocked by 1 μM ethidium bromide, as well as at the higher concentrations (not shown); the enzyme induction by isoproterenol was relatively insensitive to the inhibitor, about 60 per cent being blocked in 24 hr by 6 μM ethidium bromide. These data are included in this report because they were reproducibly observed, but we have no simple explanations for such widely diverse responses to ethidium bromide in the presence of these three 'types' of mono-oxygenase inducers.

Also in Fig. 5, 1 or 2 μM ethidium bromide has very small (10–20 per cent) effects on gross RNA synthesis over the 24-hr period. Yet gross protein synthesis is blocked about 50 and 70 per cent, respectively, by the end of the 24-hr experiment.

Puromycin aminonucleoside effects. Puromycin aminonucleoside preferentially inhibits rRNA synthesis [12, 31–33] and cytoplasmic mRNA appearance [22], presumably acting as a competitive analog of adenosine and adenine, and has no direct effect on gross protein synthesis [34, 35]. Figure 6 shows that hydroxylase induction by phenobarbital is significantly blocked by concentrations of puromycin aminonucleoside between 1 and 100 μM. The induction process by benz[a]anthracene and isoproterenol, on the other hand, is essentially unaffected by 10 and 100 μM concentrations of puromycin aminonucleoside respectively. A transient effect on both gross RNA and protein syntheses was found with 1 μM puromycin aminonucleoside: 100 μM puromycin aminonucleoside persistently inhibited gross RNA synthesis by at least 50 per cent for the 24-hr experiment, whereas little effect on gross protein synthesis was seen.

At levels of puromycin aminonucleoside greater than 100 μM, we observed paradoxically less inhibition of hydroxylase induction by benz[a]anthracene, phenobarbital or isoproterenol. This could be explained (Fig. 7) by our discovery that puromycin aminonucleoside itself is an inducer at concentrations between 0·5 and 5·0 mM. In fact, the hydroxylase activity in Hepa-1 cells treated with 5·0 mM puromycin aminonucleoside was induced in 24 hr to about the

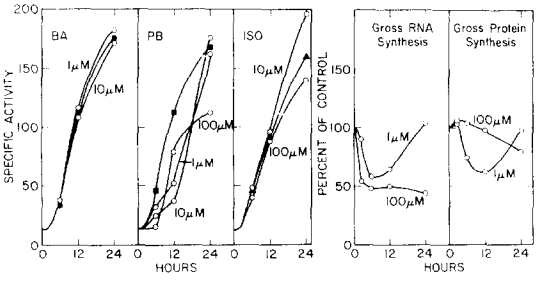

Fig. 6. Effect of puromycin aminonucleoside on hydroxylase induction and on gross RNA and protein synthesis in Hepa-1 cultures. Inducing concentrations and the abbreviations for benz[a]anthracene (●), phenobarbital (■) and isoproterenol (▲) are the same as those in Fig. 4. Concentrations of 1·0, 10 and 100 μM puromycin aminonucleoside in the presence of each inducer are shown with open symbols. Puromycin aminonucleoside alone at these concentrations had no effect on the control enzyme activity. Gross RNA synthesis (i.e. [^{14}C]uridine incorporation) and gross protein synthesis (i.e. [^{3}H]phenylalamine synthesis) are shown at right.

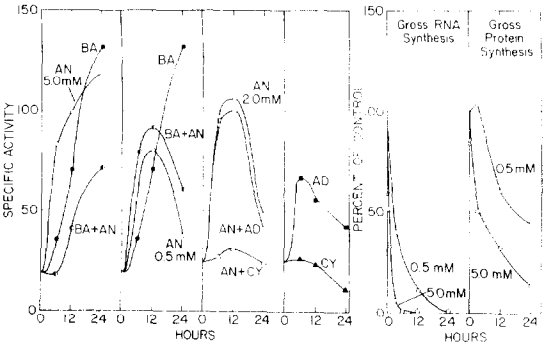

Fig. 7. Induction of hydroxylase activity in Hepa-1 cultures by high concentrations of puromycin aminonucleoside (AN) in the presence or absence of 1·3 μM benz[a]anthracene (BA). The effects of 40 nM actinomycin-D (AD) and 350 nM cycloheximide (CY), alone or in combination with puromycin aminonucleoside, are shown in the third and fourth frames. The effects of 0·5 and 5·0 mM puromycin aminonucleoside on gross RNA synthesis (i.e. [^{14}C]uridine incorporation) and gross protein synthesis (i.e. [^{3}H]phenylalamine incorporation) are shown at right.

same level as that induced by benz[a]anthracene, in spite of the fact that gross RNA synthesis was totally blocked and gross protein synthesis was more than 80 per cent inhibited. After maximal induction of the hydroxylase activity by phenobarbital or benz[a]anthracene, addition of cycloheximide plus the aminonucleoside did not impede the normal rate of hydroxylase decay (data not illustrated). This result differs from that when benz[a]anthracene, phenobarbital or norepinephrine combined with cycloheximide is added to cells containing fully induced hydroxylase activity [3, 4]. The third frame of Fig. 7 shows that the induction process by 2·0 mM puromycin aminonucleoside is not sensitive to actinomycin-D but is readily sensitive to cycloheximide; this suggests the dependence on newly synthesized protein but not dependence on newly synthesized RNA, in order for the hydroxylase induction by puromycin aminonucleoside to occur. Puromycin aminonucleoside is the first compound we have encountered that has the capacity to 'induce' the hydroxylase activity in cultured cells when actinomycin-D is added simultaneously with the 'inducer'. The simultaneous exposure of cells to a combination of 2·0 mM aminonucleoside plus cycloheximide results in no enzyme induction. This result is consistent with all data from previous studies: that the hydroxylase induction process cannot proceed any time that more than 95 per cent of the total gross protein synthesis is inhibited.

The fact that actinomycin-D alone causes a transient induction of hydroxylase activity (Fig. 7, fourth frame) indicates to us that actinomycin-D, like puromycin aminonucleoside, 'induces' the enzyme activity by some mechanism occurring in the nearly total absence of gross RNA synthesis. Cycloheximide alone does not cause the hydroxylase activity to rise. These data represent the first time aryl hydrocarbon hydroxylase activity has been shown to accumulate presumably without first an accumulation of putative hydroxylase-specific RNA. The hydroxylase induction occurs when fetal rat hepatocytes [3] or fetal hamster secondary cultures [36, 37] are exposed to inducer plus cycloheximide, followed by replacement of the

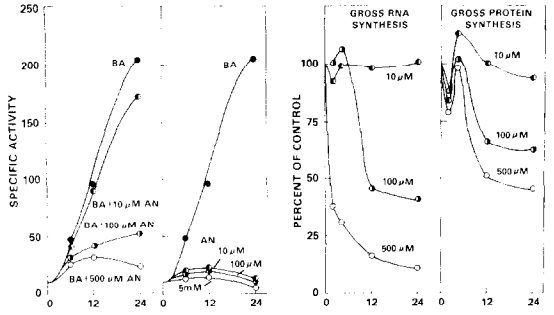

Fig. 8. Effects of low or high concentrations of puromycin aminonucleoside (AN), alone or combined with 13 μM benz[a]anthracene (BA), on the hydroxylase induction in fetal rat primary hepatocyte cultures. The effects of similar concentrations of aminonucleoside on gross RNA synthesis (i.e. [^{14}C]uridine incorporation) and gross protein synthesis (i.e. [^{3}H]phenylalamine incorporation) are shown at right.

inducer and cycloheximide with actinomycin-D. Also the hydroxylase induction occurs when rat liver-derived cells [38] are transiently exposed to cycloheximide, followed by replacement of the cycloheximide with actinomycin-D. However, these experiments differ from those illustrated in Fig. 7 in that in the latter case we gave the Hepa-1 cells no prior treatment during which an accumulation of putative mRNA might occur.

Hepa-1 is derived from a neoplasm. Does puromycin aminonucleoside also exert this dual effect on normal, nonmalignant cells in culture? Figure 8 shows that low and high concentrations of the aminonucleoside both inhibit the hydroxylase induction by benz[a]anthracene in fetal rat cultured primary hepatocytes. The concentration of aminonucleoside at which hydroxylase induction by benz[a]anthracene is only slightly affected (i.e. 10 μM) is the same in Hepa-1 cells (Fig. 6) and in these normal liver cultures (Fig. 8). The aminonucleoside alone also does not induce the hydroxylase activity. Hence, the apparent induction of enzyme activity by high concentrations of puromycin aminonucleoside in the Hepa-1 cell line is different from the result in nonmalignant cell cultures derived from normal rat liver.

DISCUSSION

In this report we have characterized, in cell lines derived from two rat hepatomas and one mouse hepatoma, the optimal conditions for hydroxylase induction: the medium and serum requirements and the inducing concentrations of a variety of hydrophobic inducers. The additive effects of phenobarbital plus a polycyclic hydrocarbon on hydroxylase induction in Hepa-1 and H-4-II-E cell lines observed in this study are similar to the additive effects of these different types of inducers previously shown in fetal rat liver primary cultures [1, 2] and in the intact animal [5]. These observations indicate that these two established cell lines, as model systems for studying aryl hydrocarbon hyroxylase induction, are not phenotypically different from normal fetal hepatocytes—in their capacity to respond via two different mechanisms to these two different types of inducers. With a biogenic amine (i.e. isoproterenol, epinephrine or tryptamine) we could not demonstrate reproducibly that this third 'class' of inducer in these established cell lines could cause additive effects with phenobarbital and/or a polycyclic hydrocarbon, as had been shown [4] in normal cultured fetal rat hepatocytes. In some experiments, however, it was clear that the biogenic amine being studied at certain concentrations did give additive effects with phenobarbital or benz[a]anthracene; thus, these established cell lines must have some potential to express the hydroxylase induction by an apparent third mechanism of enzyme induction. Extensive experimentation with different and varying concentrations of biogenic amines showed that these compounds at optimally inducing concentrations sometimes caused slight adverse morphological effects. Therefore, we believe that the difficulty in reproducibly demonstrating additive effects with any combination which included a biogenic amine may reflect subtle toxic effects not evident by light microscopy.

Distinct differences in the response of the benz[a]anthracene-, phenobarbital- or isoproterenol-mediated induction process to such metabolic inhibitors as cordycepin, ethidium bromide and puromycin aminonucleoside further suggest that the mechanisms for these three types of induction processes are indeed different. One possibility to consider is that the control, benz[a]anthracene-, phenobarbital- or isoproterenol-treated hepatoma cultures may vary with respect to the metabolism of one or more of the metabolic inhibitors used in this study. Figure 9 shows chemical structures of the compounds to which the cell cultures were exposed. The structures clearly offer possibilities for common drug-metabolizing reactions such as ring hydroxylation, hydrolysis, amine oxidation, *N*-dealkylation and glucuronidation. This possibility of metabolism may help explain, for example, the unusual effects of ethidium bromide on the hydroxylase induction by the three types of inducers (Fig. 5).

Puromycin aminonucleoside does inhibit certain small molecular weight nonhistone nuclear proteins [39] in normal human WI-38 fibroblasts but does not inhibit this class of proteins in SV_{40}-transformed WI-38 cells. In normal fetal rat cultured hepatocytes (Fig. 8), low or very high concentrations of puromycin aminonucleoside (ranging between 10 μM and 5·0 mM) both inhibited completely the hydroxylase induction by benz[a]anthracene. This difference between effects of the nucleoside seen in normal liver cells and in the neoplastic Hepa-1 cell line (Fig. 6) is in some ways similar to the difference seen by Cholon and Studzinski [22] and suggests that hydroxylase-specific mRNA synthesis, processing or stabilization is very different between Hepa-1 and normal fetal rat liver primary cultures.

At 5·0 mM concentrations, puromycin aminonucleoside is as effective as benz[a]anthracene in inducing the hydroxylase activity (Fig. 7, left frame). The large differences in the response of hydroxylase induction—seen when one varies the puromycin aminonucleoside concentration over a 1 μM–5 mM range—suggest to us at least two distinct effects of the aminonucleoside: (1) one effect secondary to inhibition of the synthesis of rRNA and/or the poly(A) segment of mRNA by low (1–100 μM aminonucleoside) concentrations, and (2) the other effect in the

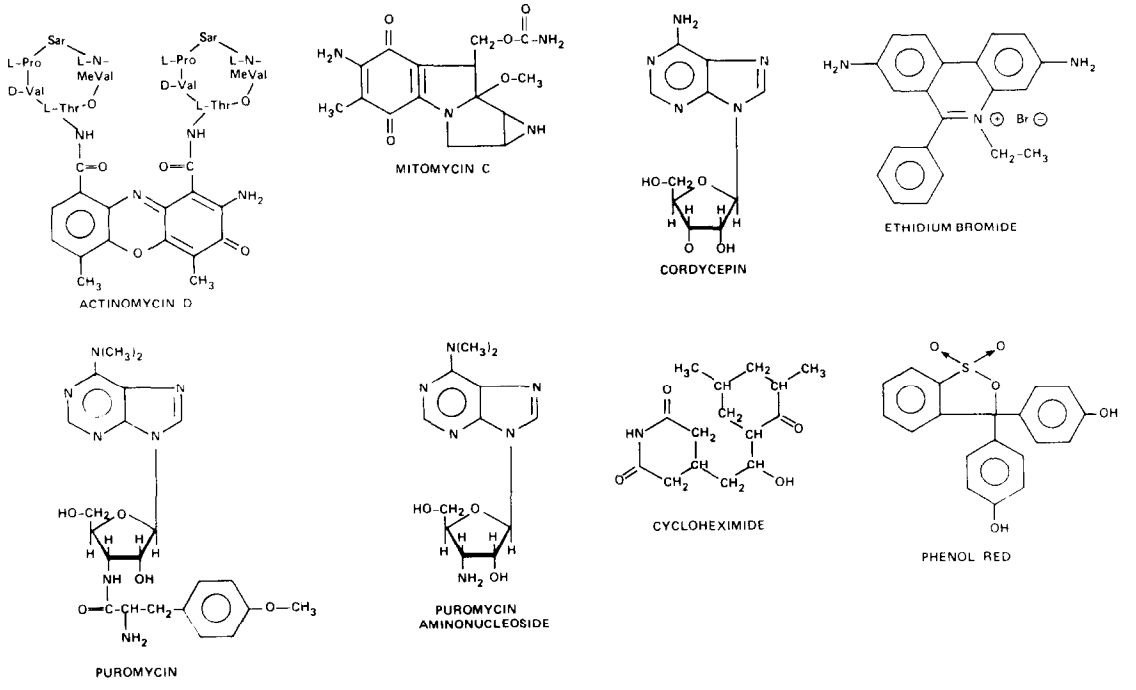

Fig. 9. Chemical structures of commonly used metabolic inhibitors, including those used in this tissue culture study. Also shown is the structure of phenol red, the commonly used pH indicator dye in many types of tissue culture growth media

nearly total absence of gross RNA synthesis by high (0·5 to 5 mM aminonucleoside) concentrations.

Hydroxylase induction by 5·0 mM puromycin aminonucleoside occurs more rapidly than that by benz[a]anthracene although gross RNA synthesis is inhibited more than 99 per cent and gross protein synthesis is inhibited 55–85 per cent during the last 18 hr of the 24-hr experiment (Fig. 7). There are several possible explanations for this 'induction' process to occur under such adverse metabolic conditions. Hepa-1 cells possess at least five times more basal hydroxylase activity than any other cells we have examined in culture; this fact supports our feeling that Hepa-1 cells have an unusually large pool of putative hydroxylase-specific RNA which is responsible for synthesis of the high basal enzyme activity. High concentrations of puromycin aminonucleoside or actinomycin-D may stabilize in some manner the existing mRNA for hydroxylase activity and allow more frequent translation of these templates to the exclusion of other mRNA's. Perhaps other mRNA species—which are far more labile than the hydroxylase mRNA species—are no longer competing for polysomes in the translational process and thus the rate of hydroxylase accumulation is stimulated by the relatively more frequent translation of the more stable hydroxylase-specific mRNA. Such a possibility may also explain the finding [40] that the rate of hydroxylase induction by aromatic hydrocarbons in various cell cultures is stimulated in the presence of interferon. Another possibility is that the large depression in gross protein synthesis results in an enlarged tRNA pool such that a crucial tRNA, which is usually rate limiting when all mRNA molecules are translated, is now available in much higher amounts. Certain steroid hormones in mammalian systems [41, 42] stimulate the synthesis of specific isoaccepting tRNA molecules simultaneously with the induction of hormonally induced proteins; these data suggest that a specific species of tRNA may be rate limiting in some control mechanism regulating protein induction. Although the hypothesis that certain tRNA levels may be rate limiting for enzyme induction has not been firmly established, it has been shown [43, 44] with cell-free protein-synthesizing systems *in vitro* that the concentration of specific tRNA can regulate the rate of mRNA translation. Thus, it is possible that rate limiting amounts of specific tRNA's regulate protein synthesis at the translational level by slowing polypeptide chain elongation or affecting initiation.

The hydroxylase induction by high concentrations of puromycin aminonucleoside or actinomycin-D may also result from a concerted action of translational initiation factors plus the diminishing mRNA pool. Induction of aryl hydrocarbon hydroxylase activity in rat liver by polycyclic hydrocarbons is associated with the stimulation of two translational initiation factors [45]; thus, an increase in synthesis, or decrease in degradation, of some initiation factor(s)—combined with a higher ratio of hydroxylase mRNA molecules to the total mRNA pool—might cause marked rises in hydroxylase activity under quite adverse metabolic conditions. Further studies to elucidate the mechanism of hydroxylase induction in liver-derived cultures treated with high doses of puromycin aminonucleoside or actinomycin-D may be important in understanding the mechanisms of such processes as drug hepatotoxicity and chemical or viral carcinogenesis.

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